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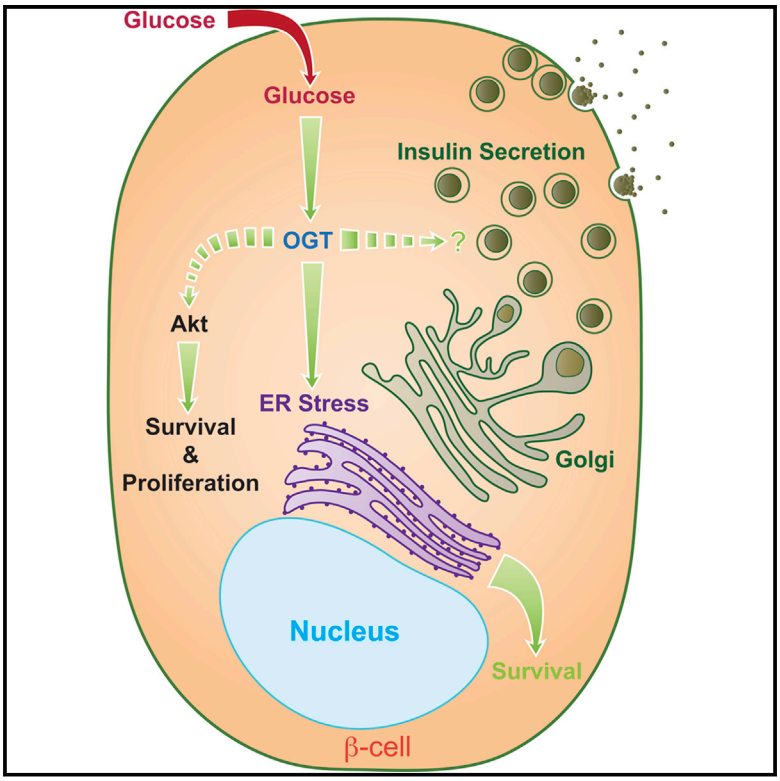
Disruption of O-linked N-Acetylglucosamine Signaling Induces ER Stress and β Cell Failure

Graphical Abstract



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In Brief

Aberrant O-GlcNAcylation has been linked to diabetes. However, little is known about the role of O-GlcNAcylation in insulin-producing β cell function and survival. Alejandro et al. show that mice lacking OGT in their β cells develop diabetes and β cell failure because of increased ER-stress-induced apoptosis and decreased Akt-induced proliferation.

Highlights

- Mice lacking β cell OGT develop diabetes and β cell failure
- OGT-deficient β cells have increased ER-stress-induced apoptosis
- OGT-deficient β cells have decreased Akt-driven proliferation
- OGT-deficient β cells have reduced calcium signaling and insulin content

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Disruption of O-linked N-Acetylglucosamine Signaling Induces ER Stress and β Cell Failure

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SUMMARY

Nutrient levels dictate the activity of O-linked N-acetylglucosamine transferase (OGT) to regulate O-GlcNAcylation, a post-translational modification mechanism to "fine-tune" intracellular signaling and metabolic status. However, the requirement of O-GlcNAcylation for maintaining glucose homeostasis by regulating pancreatic β cell mass and function is unclear. Here, we reveal that mice lacking β cell OGT (βOGT-KO) develop diabetes and β cell failure. βOGT-KO mice demonstrated increased ER stress and distended ER architecture, and these changes ultimately caused the loss of β cell mass due to ER-stress-induced apoptosis and decreased proliferation. Akt1/2 signaling was also dampened in βOGT-KO islets. The mechanistic role of these processes was demonstrated by rescuing the phenotype of βOGT-KO mice with concomitant Chop gene deletion or genetic reconstitution of Akt2. These findings identify OGT as a regulator of β cell mass and function and provide a direct link between O-GlcNAcylation and β cell survival by regulation of ER stress responses and modulation of Akt1/2 signaling.

INTRODUCTION

Type 2 diabetes (T2D) occurs when pancreatic β cells fail to adequately expand and function sufficiently when there is increased insulin demand with insulin resistance (Alejandro et al., 2015). The ability of β cells to fulfill the demand in insulin is dependent on both β cell mass and function. Although the pathogenesis of β cell dysfunction in T2D remains controversial, failure of β cell mass and function has been attributed to multiple factors including endoplasmic reticulum (ER) stress (Eizirik et al., 2008; Ferrannini, 2010). Deficits in adaptive β cell mass are partly attributed to enhanced apoptosis in T2D (Marchetti et al., 2010) and inherent poor self-renewal capabilities of β cells (Dor et al., 2004; Teta et al., 2007).

During the pathogenesis of T2D, β cells are continually exposed to high levels of glucose. Approximately 3%–5% of glucose entering the β cell is shunted to the hexosamine biosynthetic pathway (HBP) for the synthesis of uridine diphosphate-N-acetylglucosamine (UDP-GlcNAc), the substrate for O-GlcNAcylation, a dynamic and reversible post-translational protein modification analogous to phosphorylation that affects the function, stability, and sub-cellular localization of β cell proteins (i.e., Pdx-1). O-GlcNAcylation involves the addition of a single O-GlcNAc to serine or threonine residues on nuclear and cytosolic proteins solely by the enzyme O-linked GlcNAc transferase (OGT). The O-GlcNAc is removed by the O-linked β-N-acetyl hexosaminidase (O-GlcNAcase or OGA) enzyme. O-GlcNAcylation has been implicated in the etiology of insulin resistance and glucose toxicity in diabetes (Yang et al., 2008; Copeland et al., 2008). Variants in OGA are associated with T2D (Lehman et al., 2005), suggesting the importance of O-GlcNAcylation in human diabetes. However, it is poorly understood how O-GlcNAcylation modulates β cell responses to affect diabetes.

OGT is encoded by a single gene on the X chromosome and is critical for development because whole-body deletion of OGT is embryonically lethal (Shafi et al., 2000). Although β cells uniquely express high levels of OGT, the importance of O-GlcNAcylation in insulin-secreting cells is unclear. O-GlcNAcylation of critical insulin signaling proteins to regulate metabolism (IRS-1, PDK1, Akt1/2, and FoxO1) and transcription factors required for insulin biosynthesis (NeuroD1, Pdx-1, and MafA) have been demonstrated, implying the importance of O-GlcNAcylation by OGT in β cells (Andrali et al., 2007; Gao et al., 2003). Filholaud et al. demonstrated that O-GlcNAcylation is also essential for β cell development in vitro (Filhoulaud et al., 2009). The effect of decreased O-GlcNAcylation on β cell

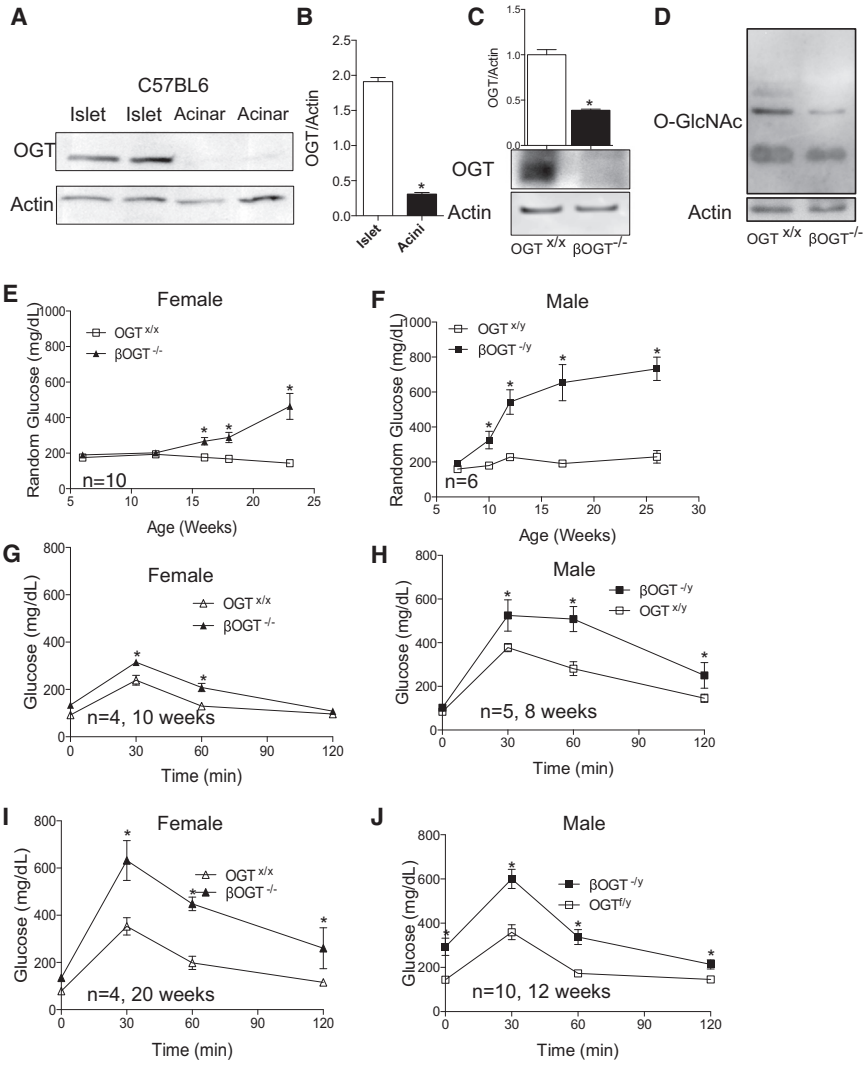

Figure 1. Conditional Deletion of OGT in β Cells Causes Severe Hyperglycemia and Glucose Intolerance in an Age-Dependent Manner

(A and B) OGT protein level in primary mouse islet and acinar (A), and quantification is shown in (B).

(C) OGT protein level and quantification in islets from 8-week-old $\beta OGT^{-/-}$ mice.

(D) O-GlcNAc level, a readout of OGT activity, in islets from 8-week-old $\beta OGT^{-/-}$ and control mice.

(E and F) Development of hyperglycemia in female (E) or male (F) βOGT-KO mice compared to control.

(G–J) Intraperitoneal glucose tolerance test (IPGTT) was performed in female (G and I) and male (H and J) βOGT-KO mice and littermate control. *$p < 0.05$ versus βOGT-KO and control.

function has been tested by overexpressing OGA in β cells: these transgenic mice have increased glucose intolerance, decreased insulin synthesis, and secretory capacity only at young age (Soesanto et al., 2011). However, the mechanisms driving these phenotypes remain unknown.

The present studies investigate the direct requirement of O-GlcNAcylation in regulating β cell mass and function in vivo. Using multiple genetic approaches, we report that female and male mice harboring β cell OGT deletion ($\beta OGT^{-/-}$ or $\beta OGT^{-/y}$, respectively) developed severe hyperglycemia, glucose intolerance, and impaired insulin secretion, and these mice ultimately exhibit severe diabetes due to β cell failure. Loss of β cell mass due to enhanced apoptosis was preceded by ER stress and downregulation of critical prosurvival proteins Akt1/2 and Pdx-1. Deletion of one Chop allele, a protein induced by ER stress, improves hyperglycemia and restores β cell mass in $\beta OGT^{-/y}$ confirming the critical mechanistic role of ER stress in the loss of β cells. Finally, the rescue of the metabolic dysfunction in $\beta OGT^{-/y}$ mice overexpressing of Akt2 [$\beta OGT^{-/-}$;caAkt(Tg)] highlights the importance of Akt signaling in islets lacking OGT. Together, these data implicate that loss of OGT leads to deterioration of β cell mass, defects in insulin secretion, and diabetes. These data identify OGT as a regulator of β cell mass and function and emphasize the emerging role of this protein in β cell survival by modulating ER stress and Akt signaling.

RESULTS

Conditional Deletion of OGT in β Cells Causes Severe Hyperglycemia in an Age-Dependent Manner

Assessment of OGT expression in acinar and islet lysates demonstrated that this protein is expressed primarily in islets (Figures 1A and 1B). To identify the importance of OGT in β cell mass and function, we generated mice with conditional deletion of OGT in β cells (male RIPCre;$OGT^{flox/y}$ ($\beta OGT^{-/y}$) or female RIPCre;$OGT^{flox/flox}$ ($\beta OGT^{-/-}$) by crossing male mice expressing Cre recombinase in β cells (RIPCre) (Herrera, 2000) with female $OGT^{flox/flox}$ (breeding scheme shown in Figure S1A). Efficient and specific recombination of the RIPCre enzyme in β cells of $\beta OGT^{-/y}$;CAG^{GFP} (with GFP reporter) mice was observed by EGFP expression in the majority of the β cells (Figure S1B). OGT protein and mRNA levels were significantly reduced in islets from $\beta OGT^{-/-}$ mice compared to control ($OGT^{x/x}$; Figure 1C; Figure S1C). A reduction in OGA mRNA level was also observed (data not shown). No alteration of OGT protein level was observed in hypothalamus and liver tissues (Figures S1D and S1E). A reduction of O-GlcNAcylation of proteins in islets from $\beta OGT^{-/-}$ mice confirmed OGT deletion in β cells (Figure 1D). Body weight in male $\beta OGT^{-/y}$ and female $\beta OGT^{-/-}$ mice was comparable to littermate control ($OGT^{x/y}$ or $OGT^{x/x}$ data not shown). Examination of female $\beta OGT^{-/-}$ and littermate control mice ($OGT^{+/+}$, here in referred to as control) showed hyperglycemia by 16 weeks of age (Figure 1E). In contrast,

βOGT$^{-/y}$ mice developed hyperglycemia earlier than female βOGT$^{-/-}$ with glucose levels greater than 350 mg/dl by 10 weeks (Figure 1F). A reduction in fed insulin levels was observed in 10-week-old βOGT$^{-/-}$ mice (data not shown). These studies demonstrate that male and female mice lacking OGT in β cells develop severe hyperglycemia in an age-dependent manner.

Young and Adult Mice with Conditional Deletion of OGT in β Cells Develop Glucose Intolerance

Next, we assessed glucose homeostasis in young and adult mice lacking OGT in β cells. We observed significant impairment in glucose tolerance in female βOGT$^{-/-}$ mice starting at 10 weeks, a time point where they were still normoglycemic (Figures 1E and 1G). These mice developed severe glucose intolerance with elevated fasting blood glucose level by 20 weeks of life (Figure 1I). Because the male βOGT$^{-/y}$ mice developed hyperglycemia at earlier age, we tested glucose tolerance in 4-week-old males. These mice demonstrated normal glucose tolerance compared to control (Figure S1F). In contrast, 8-week-old βOGT$^{-/y}$ male mice exhibited glucose intolerance without fasting hyperglycemia compared to control (Figure 1H). Impaired glucose tolerance was even more significant with elevated fasting glucose level by 12 weeks of age (Figure 1J). Together, these data show young male and female mice lacking OGT present glucose intolerance without apparent alteration in random glucose level.

Young Mice Lacking OGT in β Cells Exhibit Normal β Cell Mass while Adult Mice Show Reduced β Cell Mass

Next, we sought to assess the relative contribution of insulin sensitivity in peripheral tissues. Despite demonstrating glucose intolerance, 8-week-old male βOGT$^{-/y}$ mice presented normal insulin tolerance (Figure 2A), pointing to a defect in β cell mass or insulin secretion. To tease apart the relative contribution of β cell mass and function, we first assessed β cell mass in various ages. Because we observed similar phenotype in both genders, we focused on male βOGT$^{-/y}$. We observed no alteration in β cell mass at 4- and 7-week-old βOGT$^{-/y}$ mice compared to control (Figures 2B and 2C). Examination of adult 12-week-old βOGT$^{-/y}$ mice exhibited significant reduction in β cell mass (Figure 2D). Adult female βOGT$^{-/-}$ mice also demonstrated reduced β cell mass (Figure S2A). The reduction in β cell mass at 3 months was associated with a significant decrease in proliferation (Figure 2E), as well as induction of β cell death measured by TUNEL staining (Figure 2F). Not surprisingly, significant reduction in β cell mass was associated with blunted insulin secretion in 12-week-old βOGT$^{-/y}$ mice compared to control (Figure 2G). Thus, significant reduction in β cell mass was only observed in adult mice harboring OGT deletion in β cells.

Young Mice Lacking OGT in β Cells Have a Defect in Insulin Secretion, in Part Due to Altered Ca^{2+} Signaling

To identify an independent β cell function defect, islets from 8-week-old normoglycemic βOGT$^{-/-}$ mice with normal β cell mass were assessed for insulin secretion in vitro. We observed diminished insulin secretion in response to both high glucose and KCl (Figure 2H) in βOGT$^{-/-}$ islets. Because we detected reduced insulin secretion in response to KCl, we assessed Ca^{2+} signaling and discovered that it was impaired in islets from 8-week-old normoglycemic βOGT$^{-/-}$ mice (Figure 2I). Interestingly, approximately 33% of βOGT$^{-/-}$ islets did not oscillate in response to 8 mM glucose compared to control (where 100% of islets oscillated). The oscillations of βOGT$^{-/-}$ islets showed reduced amplitude, period, and plateau fraction in response to 11 mM glucose compared to control (Figures 2J–2M). Assessment of time-dependent changes in calcium signaling showed impaired Ca^{2+} signaling at different ages including normoglycemic 6-week-old mice and hyperglycemic 19-week-old mice lacking OGT (Figure S2B). In addition to altered Ca^{2+} signaling, we identified that insulin content was reduced in normoglycemic βOGT$^{-/y}$ and βOGT$^{-/-}$ mice (Figures S3A and S3B). A reduction in insulin content can be partly explained by the downregulation of *ins1*, *ins2*, *Pdx-1*, and *NueroD1* mRNA levels in islets from male and female mice lacking OGT (Figures S3C–S3I). These data implicate reduced Ca^{2+} signaling and insulin content as contributing factors to defective insulin secretion in mice with conditional deletion of OGT.

Mice with Inducible Deletion of OGT in Mature β Cells Develop Glucose Intolerance and Defective Insulin Secretion

To assess the requirement of OGT in mature β cells, we deleted OGT in β cells of 12-week-old mice. By breeding the inducible MIPCre-ERTM and OGT$^{flox/flox}$, we generated inducible MIPCre-ERTM;OGT$^{-/y}$ mice (iβOGT$^{-/y}$). The MIPCre-ERTM recombination was efficient after tamoxifen (TMX) injection as demonstrated by β cell-specific expression of GFP reporter transgene (Rosa-CAGZsGreen) only in iβOGT$^{-/y}$ islets (Figure S3J). Four weeks after TMX injection, we observed ~70% β cells expressing the GFP reporter both in iβOGT$^{-/y}$ mice and control mice (MIPCre-ERTM; CAG-ZsGreen, Figure S3K). iβOGT$^{-/y}$ mice did not develop hyperglycemia compared to βOGT$^{-/y}$ mice (data not shown). Eight weeks post-TMX injection resulted in moderate but significant glucose intolerance in iβOGT$^{-/y}$ mice that worsened with age (Figures 3A and 3B). Significant glucose intolerance was observed at 12 and 22 weeks post-OGT ablation. In parallel to the glucose intolerance, an insulin secretion defect was detected in iβOGT$^{-/y}$ mice by glucose-stimulated insulin secretion (GSIS) test in vivo, confirming the importance of OGT in insulin secretion (Figure 3C). No significant changes in β cell mass were observed between iβOGT$^{-/y}$ and control mice at 22 weeks post-TMX injection (Figure 3D). These data from the inducible model clearly suggest that OGT in mature β cells is important for regulating insulin secretion.

Phloridzin Partially Ameliorates the Development of Diabetes in Mice Lacking OGT in β Cells

Because a rapid loss in β cell mass from 4 to 12 weeks old in βOGT$^{-/y}$ and not in iβOGT$^{-/y}$ mice was observed, we hypothesized that glucotoxic conditions could play a role on the decline of β cells. To assess an independent effect of glucotoxicity contributing to β cell mass loss, we treated βOGT$^{-/y}$ mice with Phloridzin (PHZ), a naturally occurring metabolite that promotes glucose excretion in the kidney via inhibition of sodium-glucose contransporters (Ehrenkranz et al., 2005). We established two cohorts of mice implanted with osmotic pumps containing

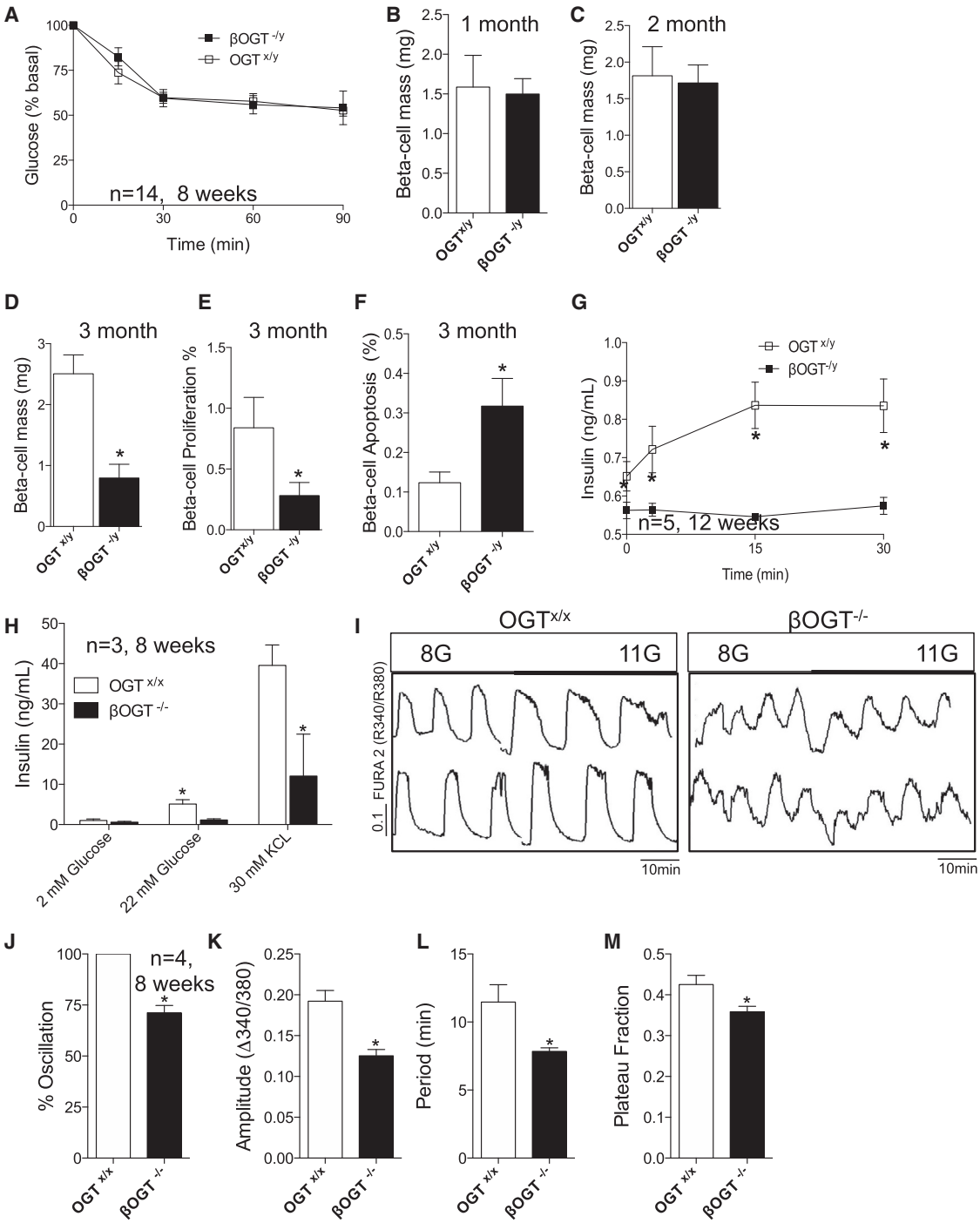

Figure 2. Young Mice Lacking OGT in β Cells Exhibit Normal β Cell Mass and Insulin Secretion Defect, While Adult Mice Show Reduced β Cell Mass

(A) Intraperitoneal insulin tolerance test (ITT) was performed in 10-week-old $\beta OGT^{-/y}$ mice and littermate control.

(B–D) β cell mass at 4- (B), 7- (C), and 12-week-old (D) $\beta OGT^{-/y}$.

(E and F) Proliferation assessed by KI67 staining (E), and apoptosis measured by TUNEL staining (F) in 12-week-old $\beta OGT^{-/y}$ mice.

(G) In vivo glucose-stimulated insulin secretion (GSIS) in 12-week-old $\beta OGT^{-/y}$ and control mice.

(H) Insulin secretion corrected to DNA content in response to 2 mM, 22 mM glucose, and 30 mM KCl of islets isolated from normoglycemic 8-week-old $\beta OGT^{-/-}$ mice and control.

(I–M) Measurement of intracellular Ca^{2+} in islets of 8-week-old $\beta OGT^{-/-}$ mice. *$p < 0.05$ versus βOGT-KO and control; n values and age of mice are noted within figures.

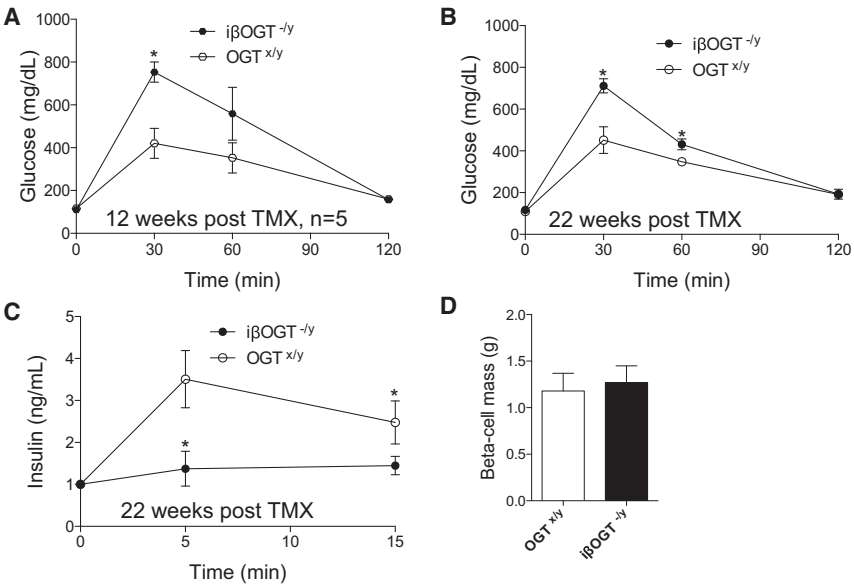

Figure 3. Mice with Inducible Deletion of OGT in Mature β Cells Develop Glucose Intolerance and Insulin Secretion Dysfunction

(A and B) Ablation of OGT in mature β cells caused glucose intolerance and insulin secretion dysfunction. IPGTT was performed in male iβOGT$^{-/y}$ (MIPCre;OGT$^{-/y}$) post-tamoxifen treatment at 12 (A) and 22 weeks (B).

(C) In vivo GSIS in iβOGT$^{-/y}$ and control at 22 weeks post-tamoxifen treatment.

(D) β cell mass assessment between iβOGT$^{-/y}$ and control mice measured at 22 weeks post-tamoxifen treatment. *$p < 0.05$ versus iβOGT$^{-/y}$ and control, n = 4–6.

PHZ. The first cohort was 10-week-old βOGT$^{-/y}$ and control mice. Prior to implantation, we observed glucose intolerance and elevated fasting blood glucose level in βOGT$^{-/y}$ mice compared to control mice (Figure S4A). No effect on body weight was observed in all mice treated with PHZ (data not shown). We tested the efficiency of PHZ to lower glucose during a fasting state on day 18 of treatment. After a 12-hr fasting, glucose levels of βOGT$^{-/y}$ mice were normalized to control (Figure S4B), suggesting PHZ was effective in reducing blood glucose levels. Fed glucose levels in βOGT$^{-/y}$ mice were reduced and remained comparable to control mice from day 2 to day 15 of PHZ treatment (Figure S4C). However, the effect of PHZ was transient; βOGT$^{-/y}$ mice eventually redeveloped hyperglycemia and glucose intolerant on day 20 of PHZ treatment (Figures S4C and S4D). When compared to age-matched βOGT$^{-/y}$ mice without PHZ treatment (Figure 1I; Figure S6D), PHZ treatment improved basal fasting glucose levels at time 0 and partially improved glucose tolerance in βOGT$^{-/y}$ mice (Figures S4E and S4F). Both fed and fasting insulin levels remained significantly reduced in βOGT$^{-/y}$ mice compared to control treated with PHZ (Figures S4G and S4H). These data suggest that hyperglycemia contributed to the metabolic dysfunction in βOGT$^{-/y}$ mice. Because PHZ normalized fasting blood glucose levels in βOGT$^{-/y}$ mice treated with PHZ and glucose intolerance was still present at this time, these data point to an inherent insulin secretion defect in mice lacking OGT.

We established a younger cohort of mice to assess whether an earlier and longer period of intervention could rescue the metabolic dysfunction in βOGT$^{-/y}$ mice. Seven-week-old βOGT$^{-/y}$ mice and control were implanted with ALZET pumps with PHZ. Fed glucose levels of βOGT$^{-/y}$ mice treated with PHZ were comparable to control up to 16 days of continuous treatment (Figure 4A). When comparing to age-matched mice without PHZ treatment, βOGT$^{-/y}$ mice treated with PHZ demonstrated improved fed glucose levels (Figure 4B). On day 17 of PHZ treatment, βOGT$^{-/y}$ mice showed improved glucose tolerance that was not different than that of the control (Figure 4C). Comparing βOGT$^{-/y}$ mice with or without PHZ, the mice treated with PHZ were metabolically better (Figures 4D and 4E). However, PHZ treatment was insufficient to maintain glucose tolerance in βOGT$^{-/y}$ mice on day 25 of treatment (Figure 4F). Although βOGT$^{-/y}$ mice treated with PHZ eventually developed glucose intolerance compared to control with PHZ, they remained metabolically better compared to βOGT$^{-/y}$ mice not treated with PHZ (Figure 4E). No differences in body weight or fed or fasting insulin levels were observed (Figures 4G and 4H). Despite the transient improvement in glucose tolerance, no restoration of β cell mass was observed in β cell mass in βOGT$^{-/y}$ mice treated with PHZ (Figure 4I). Interestingly, PHZ treatment restored β cell proliferation and apoptosis in βOGT$^{-/y}$ mice to control levels (Figures 4J and 4K). Together these data suggest that PHZ intervention was beneficial in the younger cohort of βOGT$^{-/y}$ mice and delayed the onset of diabetes. However, despite improving glucose levels and normalizing proliferation and apoptosis to control levels, PHZ did not restore β cell mass in βOGT$^{-/y}$ mice. Thus, it may require an earlier intervention or longer PHZ treatment to observe a major impact on β cell mass. It is also possible that PHZ treatment only reduces the stress on β cells, improving both proliferation and survival, but not sufficient to rescue and/or restore β cell mass.

Downregulation of Prosurvival Akt1/2 and Pdx-1 Signaling and Enhanced ER Stress in Young and Normoglycemic Mice Lacking OGT in β Cells

To unravel the cause of β cell dysfunction in mice with lacking OGT in β cells, we focused our mechanistic studies on young and normoglycemic animals. The PI3K/Akt/Pdx-1 signaling is one of the major pathways regulating β cell survival, both apoptosis and ER stress (Sachdeva et al., 2009). Because Akt phosphorylation has been shown to be regulated by OGT, we assessed the phosphorylation status of prosurvival protein Akt1/2 at Ser473 in islets from normoglycemic βOGT$^{-/-}$ mice. We found a reduction in phosphorylated Akt1/2 Ser473 with increased total Akt1/2 protein level (Figures 5A–5B′). Pdx-1, a critical transcription factor for insulin gene transcription and β cell survival, was reduced in 7-week-old female βOGT$^{-/-}$ islets compared to control (Figure 5C), suggesting

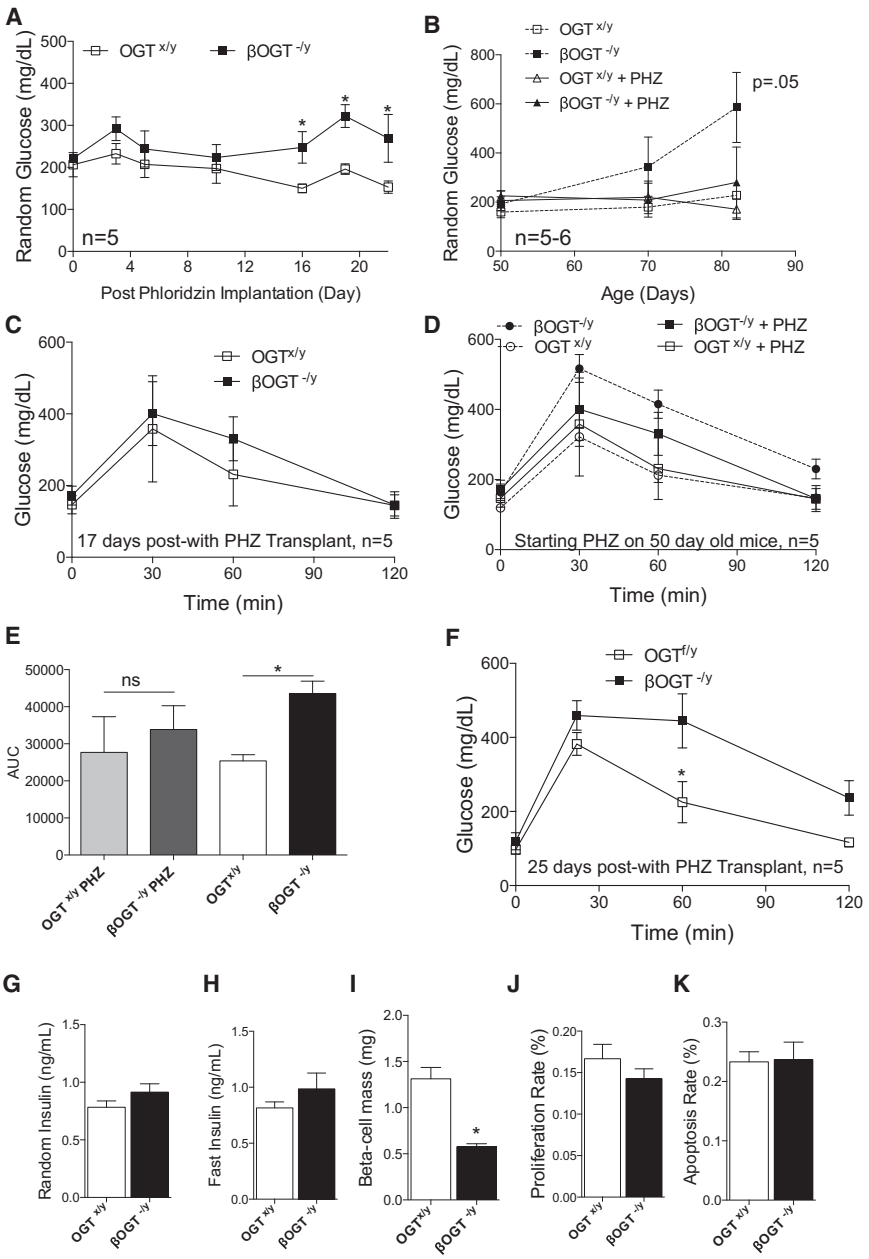

Figure 4. Phloridzin Partially Ameliorates the Development of Diabetes in Mice Lacking OGT in β Cells

(A) Random glucose levels in $\beta OGT^{-/y}$ and control mice treated with Phloridzin (PHZ).

(B) Comparison of random glucose levels in $\beta OGT^{-/y}$ mice treated with or without PHZ.

(C and D) IPGTT was performed on day 17 of PHZ (C) or without treatment (D).

(E) Quantification of area under curve (AUC) of (D).

(F) IPGTT was performed on day 25 of PHZ.

(G and H) Random (G) and fasting (H) insulin levels taken on day 20 of PHZ treatment.

(I–K) β cell mass (I), proliferation (J, measured by KI67), and apoptosis (K, assessed by TUNEL staining) between $\beta OGT^{-/y}$ and control mice on day 27 of PHZ treatment. *$p < 0.05$ versus $\beta OGT^{-/y}$ and control, n = 5.

that Pdx-1 protein levels were altered before the loss in β cell mass.

Despite normal glucose tolerance and β cell mass, 4-week-old $\beta OGT^{-/y}$ mice presented reduced insulin and elevated proinsulin circulating levels (Figures S5A–S5C), suggesting alterations in ER function. Co-staining with ER marker Calnexin showed increased accumulation of proinsulin in the ER (Figures S5D and S5E), supporting the notion that $\beta OGT^{-/y}$ mice have enhanced ER stress prior to development of glucose intolerance and β cell failure. Elevated protein expression of ER stress markers BiP, Chop, and phosphorylated EIF2α (Figures 5E–5G) were observed in islets from 4-week-old $\beta OGT^{-/y}$ mice. Increased caspase12, a specific mediator of ER-stress-induced apoptosis (Yoneda et al., 2001) was evident in islets of $\beta OGT^{-/y}$ mice (Figure 5H). Increased BiP levels were also observed in female $\beta OGT^{-/-}$ islets (Figure S5F). Furthermore, ER morphology by electron microscopy showed distended ER in islets from 4-week-old $\beta OGT^{-/y}$ mice and abnormalities in mitochondria morphology compared to control (Figure 5I). Together, these data suggest that ER stress is directly involved in the early deterioration of β cells before the onset of glucose intolerance and hyperglycemia in mice lacking OGT.

Akt Overexpression Prevents Hyperglycemia and Improves Glucose Tolerance by Normalizing β Cell Mass in Mice Lacking OGT in β Cells

Prosurvival Akt signaling was reduced in islets of 8-week-old $\beta OGT^{-/-}$ mice; thus, we hypothesized that Akt overexpression in β cells ameliorates the metabolic phenotype of mice lacking OGT. To directly assess the involvement of Akt, we generated transgenic mice overexpressing Akt [CaAkt(Tg)] (Bernal-Mizrachi et al., 2001) with β cell-specific deletion of OGT [$\beta OGT^{-/-}$;CaAkt(Tg)]. The onset of hyperglycemia was prevented in $\beta OGT^{-/-}$ mice overexpressing Akt [$\beta OGT^{-/-}$;CaAkt(Tg)] compared to $\beta OGT^{-/-}$ mice (Figure 6A). We observed increased fed and fasting insulin levels in the $\beta OGT^{-/-}$;CaAkt(Tg) compared to $\beta OGT^{-/-}$ mice alone. As expected, Akt overexpressing mice showed elevated fed and fasting insulin compared to wild-type control (Figures 6B and 6C). As shown in previous cohorts, glucose intolerance was observed in 9-week-old $\beta OGT^{-/-}$ mice that worsened with age (Figure 6D). Mice lacking OGT in β cells exhibited

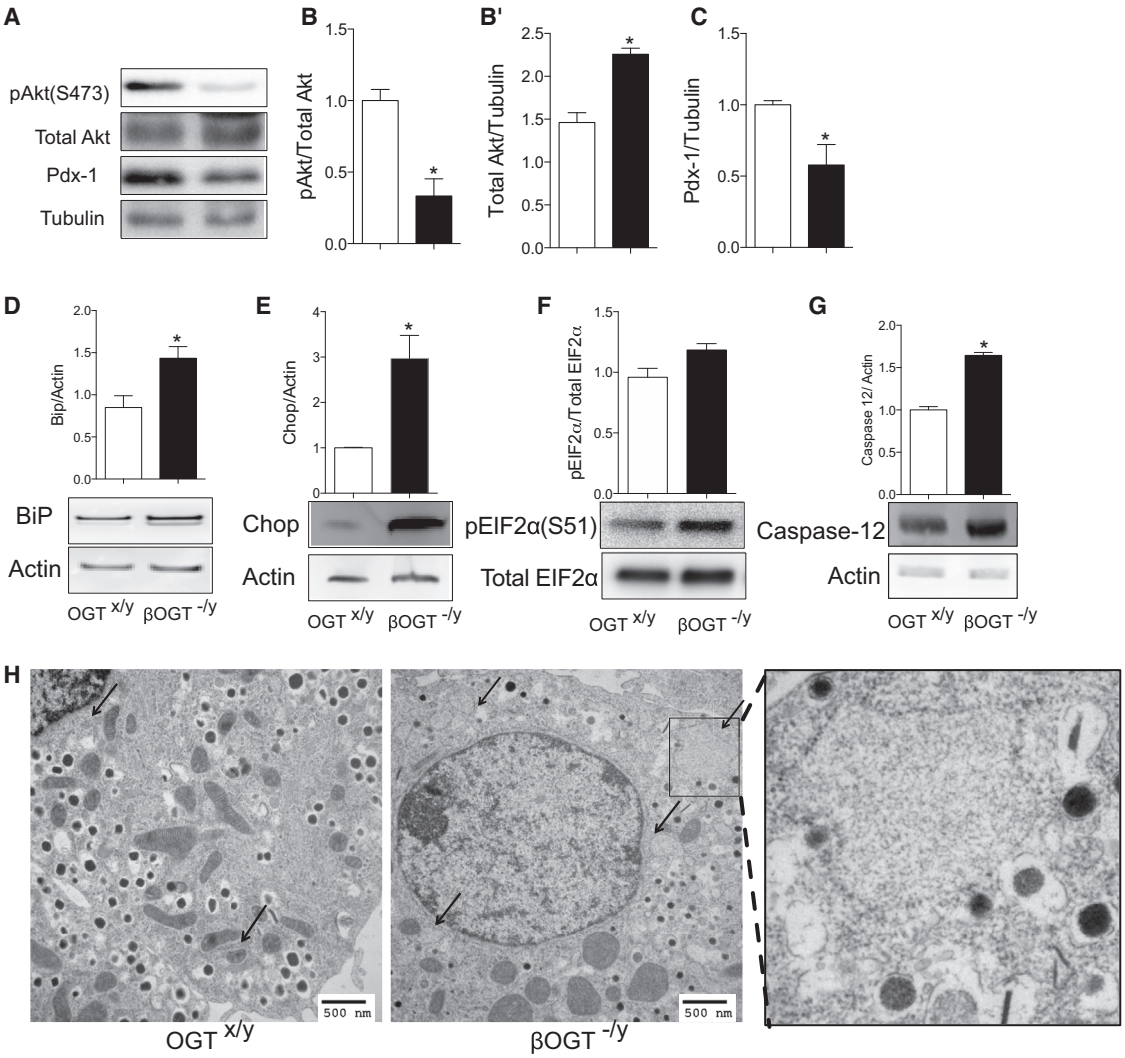

Figure 5. Downregulation of Prosurvival Akt1/2 and Pdx-1 Signaling and Enhanced ER Stress in Young and Normoglycemic Mice Lacking OGT in β Cells

(A) Downregulation of prosurvival Akt1/2 at Ser473 and Pdx-1 in islets of normoglycemic 8-week-old female $\beta OGT^{-/-}$ mice.

(B–C) Quantification phosphorylated Akt1/2(Ser473)/Total Akt one-half (B), total Akt/tubulin (B′), and Pdx-1/tubulin (C).

(D–G) ER stress markers protein expression in islets from normoglycemic 4-week-old $\beta OGT^{-/y}$ mice; BiP (D), Chop (E), phosphorylated eIF2α (F), and Caspase-12 (G) in islets of 4-week-old $\beta OGT^{-/y}$ mice.

(H) Electron microscopy images of β cell showing distended ER (arrow) and rounded mitochondria from 4-week-old $\beta OGT^{-/y}$ mice. *$p < 0.05$ versus βOGT-KO and control, n = 3.

worse glucose intolerance at 22 weeks old compared to $\beta OGT^{-/-}$;CaAkt(Tg), suggesting that overexpression of Akt improved glucose tolerance in mice lacking OGT (Figure 6E). GSIS assessment in vivo suggested that $\beta OGT^{-/-}$;CaAkt(Tg) had increased basal and stimulated insulin values compared to $\beta OGT^{-/-}$ mice (Figure 6F). β cell mass in $\beta OGT^{-/-}$;CaAkt(Tg) was significantly improved compared to $\beta OGT^{-/-}$ mice (Figure 6G). However, the increase in β cell mass in $\beta OGT^{-/-}$;CaAkt(Tg) was normalized to control and not to the CaAkt(Tg) level. We observed a robust increased in β cell proliferation in $\beta OGT^{-/-}$;CaAkt(Tg) compared to CaAkt(Tg) (Figure 6H). However, no differences in apoptosis (Figure 6I), or OGlcNAc levels were observed between islets from $\beta OGT^{-/-}$ and $\beta OGT^{-/-}$;CaAkt(Tg) even in the presence of enhanced Akt signaling in $\beta OGT^{-/-}$;CaAkt(Tg) as demonstrated by increased phosphoGSK3β (Ser9) (Figure S6A and S6B). On the other hand, we observed partial rescue of ER stress demonstrated by enhanced proinsulin localization in the Golgi of $\beta OGT^{-/-}$;CaAkt(Tg) islets compared to proinsulin staining in the ER of $\beta OGT^{-/-}$ islets (Figure S7A). These data indicate that Akt is sufficient to prevent diabetes in $\beta OGT^{-/-}$ mice by modulating β cell mass via proliferation and provide a mechanistic link between O-GlcNAcylation and β cell survival by modulation of Akt signaling.

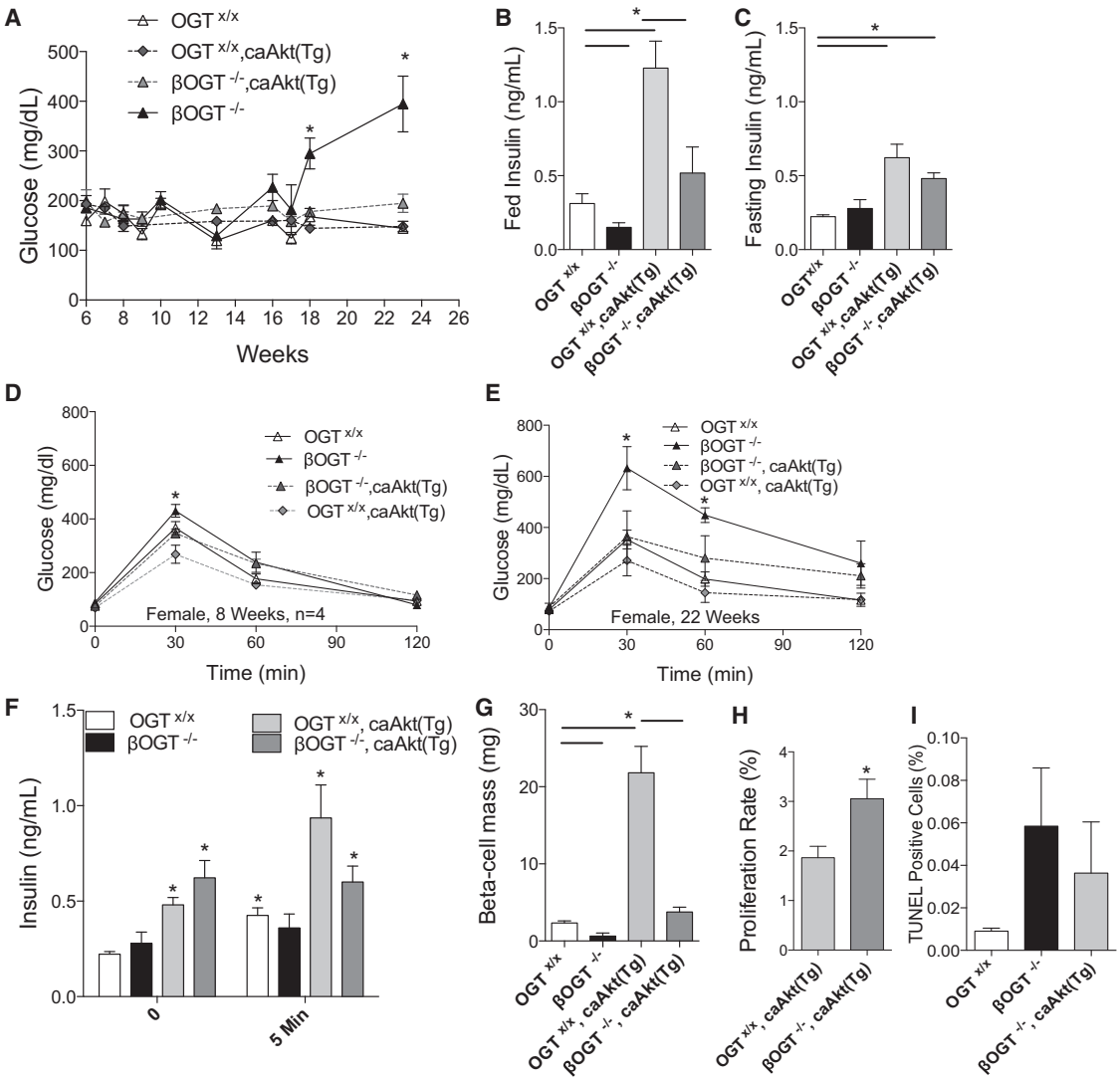

Figure 6. Akt Overexpression Prevents Hyperglycemia and Improves Glucose Tolerance in Mice Lacking OGT in β Cells

(A–C) Random glucose (A), random insulin (B), and fasting insulin (C) levels in $\beta OGT^{-/-}$ mice with or without Akt overexpression [CaAkt(Tg)].

(D and E) IPGTT was performed in 8- and 22-week-old $\beta OGT^{-/-}$ mice with [$\beta OGT^{-/-}$;CaAkt(Tg)] or without the Akt transgene [CaAkt(Tg)].

(F–I) In vivo GSIS (F), β cell mass (G), β cell proliferation (H, measured by KI67), and apoptosis (I, TUNEL) at 22-week-old $\beta OGT^{-/-}$ with or without CaAkt(Tg).

*$p < 0.05$ and $n = 4$.

Deletion of One Chop Allele Improves Hyperglycemia and β Cell Mass in Mice with Conditional Deletion of OGT in β Cells

Chronic unfolded protein response signaling leads to the commitment phase of ER-stress-induced apoptosis, where C/EBP homologous protein (Chop) can trigger pro-apoptotic signals. Having demonstrated that islets from $\beta OGT^{-/y}$ exhibited increased ER stress, we tested the hypothesis that reduction of Chop in $\beta OGT^{-/y}$ ameliorates the alterations in glucose homeostasis and loss of β cells. Indeed, deletion of one Chop allele in $\beta OGT^{-/y}$ ($Chop^{+/-};\beta OGT^{-/y}$) was sufficient to lower glucose in $\beta OGT^{-/y}$ mice (Figure 7A). Six-week-old $Chop^{+/-};\beta OGT^{-/y}$ demonstrated improved glucose tolerance compared to $Chop^{+/+};\beta OGT^{-/y}$ (Figure 7B). Fed insulin levels were also improved in 12-week-old $Chop^{+/-};\beta OGT^{-/y}$ compared to $Chop^{+/+};\beta OGT^{-/y}$ mice (Figure 7C); however, this level of insulin was not sufficient to normalize random blood glucose level. β cell mass was preserved in $Chop^{+/-};\beta OGT^{-/y}$ compared to $Chop^{+/+};\beta OGT^{-/y}$ (Figure 7D), in part, by reduction of β cell apoptosis (Figure 7E). These data suggest that ER stress plays a major role in the β cell failure in mice lacking OGT in β cells and provide a direct link between O-GlcNAcylation and β cell survival by regulation of ER stress responses.

DISCUSSION

In the present study, we define the role of O-GlcNAcylation in β cells using several genetically modified mice. We reveal that

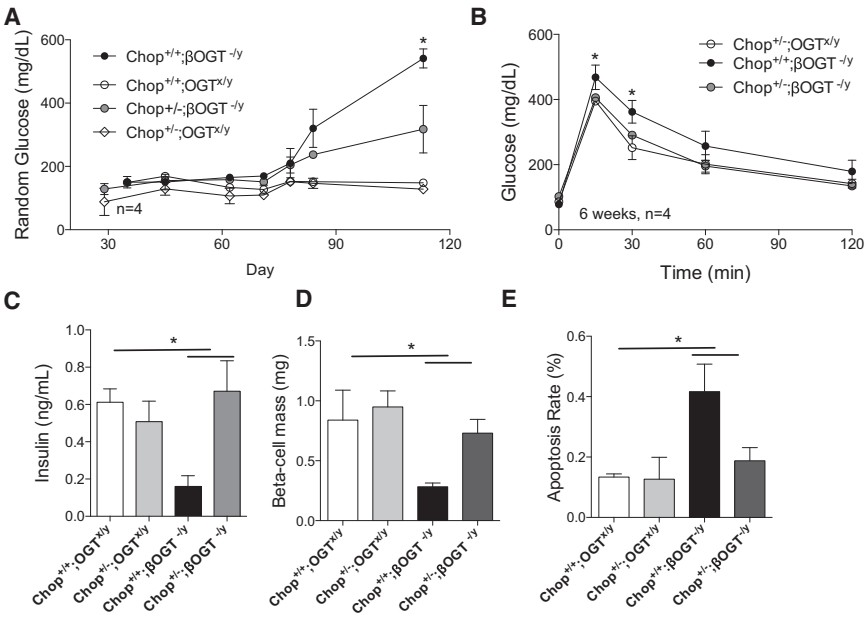

Figure 7. Deletion of One Allele of Chop Improves Hyperglycemia and β Cell Mass in Mice Lacking OGT in β Cells

(A) Random blood glucose levels taken at time points indicated.

(B) IPGTT was performed in 6-week-old βOGT$^{-/y}$ mice expressing (Chop$^{+/-}$;βOGT$^{-/y}$) or without one allele of Chop (Chop$^{+/-}$;βOGT$^{x/y}$) or wild-type control (Chop$^{+/+}$;βOGT$^{x/y}$).

(C) Blood insulin levels taken on 12-week-old mice with the shown genotypes.

(D and E) β cell mass (D) and β cell apoptosis (E, measured by TUNEL) at 12-week-old βOGT$^{-/y}$ with or without one allele of Chop. *$p < 0.05$ and $n = 4$.

OGT is indispensable for controlling both insulin secretion and β cell number. Independent of mass, OGT inherently regulates insulin secretion, partly due to alterations in calcium signaling and insulin content. In addition, we identified roles of OGT in proliferation and survival specifically through modulating Akt signaling and ER stress responses respectively. Reconstituting Akt signaling or deletion of one Chop allele to improve ER stress was sufficient to reduce hyperglycemia and glucose intolerance in βOGT$^{-/-}$ or βOGT$^{-/y}$ (βOGT-KO) mice. These findings suggest that impairment of O-GlcNAcylation leads to ER stress early in life and, subsequently, deterioration of both β cell mass and function. Together, these studies provide strong evidence of the mechanistic link between O-GlcNAcylation, ER stress, and Akt signaling in regulation of pancreatic β cell mass and function.

These studies identified the role of OGT at different stages of β cell development. OGT is dispensable for β cell development and the first 30 days of life as observed by normoglycemic and normal β cell mass. However, young mice exhibited a defect in insulin secretion and changes in Ca^{2+} signaling affecting the percentage of islets that oscillates and their profiles (amplitude, period, and plateau fraction) in response to glucose, which can all have a major impact on insulin granule release. The mechanism-mediating abnormalities in intracellular Ca^{2+} signaling are unclear, but it is possible that O-GlcNAcylation of proteins involved in Ca^{2+} handling in β cells such as VDAC, IP3 receptor, and SERCA could be involved as reported in cardiac tissue (Johnsen et al., 2013; Rengifo et al., 2007). Independent of calcium-mediated insulin secretion defect, young and normoglycemic mice lacking OGT presented lower insulin content, in part, by reduced *Ins1*, *Ins2*, *NeuroD1*, *and Pdx-1* mRNA messages. In addition, Pdx-1 O-GlcNAcylation is important for binding to the insulin gene and insulin secretion in MIN6 cells (Gao et al., 2003). The abnormalities in insulin mRNA and content are similar to those observed in transgenic mice with β cell-specific overexpression of OGA (Soesanto et al., 2011). Conversely, the current evidence support the notion that enhanced O-GlcNAcylation potentiates insulin secretion (Tang et al., 2000; Zraika et al., 2002). Taken together, previous evidence suggested that O-GlcNAcylation regulates insulin content and secretion, and our data identify an important role for O-GlcNAcylation on intracellular Ca^{2+} homeostasis. Furthermore, OGT could independently regulate cytoskeletal reorganization and proteins (i.e., synapsin I, Synaptotagmin) involved in insulin exocytosis (Cole and Hart, 1999; Fukuda, 2002).

While young mice lacking OGT in β cells exhibited normal β cell mass, adult mice demonstrated significant reduction in β cell mass because of enhanced cell death and impaired proliferation. These phenotypes were partly explained by downregulation of prosurvival Akt and Pdx-1 signaling. Akt signaling is important for β cell mass affecting both proliferation and apoptosis rates (Bernal-Mizrachi et al., 2001). Islets from βOGT-KO mice demonstrated reduced Akt phosphorylation at Ser473 compared to control. The mechanisms underlying this effect are not clear, but specific O-GlcNAcylation sites on Akt modulate Ser473 phosphorylation (Heath et al., 2014). Our data suggest that the interplay between OGT and Akt signaling is prosurvival in β cells. Overexpression of Akt in βOGT-KO mice improved glucose tolerance and prevented the onset of frank diabetes by restoring β cell mass in βOGT-KO mice suggesting that abnormalities in Akt signaling could be a major mechanism for the defects in β cell mass. Interestingly, restoration of β cell mass in βOGT$^{-/-}$;CaAkt(Tg) occurs mainly via proliferation and not survival. A potential role for Akt on rescuing apoptosis in βOGT-deficient cells cannot be ruled out as previous data have shown that Akt overexpressing mice exhibit a high β cell turnover with enhanced apoptosis and proliferation (Bernal-Mizrachi et al., 2001). It is still possible that lower levels of Akt activation could improve survival in βOGT-deficient cells and that can be tested in in vitro experiments. Finally, the cross talk between Akt and OGT signaling appears more complex as shown by the reduction of β cell mass in βOGT$^{-/-}$;CaAkt(Tg) compared to OGT$^{x/x}$;CaAkt(Tg). These results suggest that reduction of OGT signaling could modulate biological processes regulating proliferation downstream of Akt signaling.

Unlike adult mice lacking OGT since embryonic day 13.5, ablating OGT in mature β cells does not cause diabetes but, instead, glucose intolerance due to insulin secretion defect and not mass. Thus, OGT could play a protective role in states of chronic hyperglycemia and glucotoxicity and thereby regulating β cell mass and function. OGT modulates O-GlcNAcylation of proteins according to glucose level availability. Hyperglycemia increases glucose flux through HBP, as such enhancing OGT activity and O-GlcNAcylation. Non-physiological levels of glucosamine promote β cell dysfunction upon chronic exposure to hyperglycemia (Kaneto et al., 2001). In the present study, mice lacking OGT appear to be more sensitive to glucotoxic condition because PHZ treatment delayed the onset of diabetes and normalized proliferation and apoptosis rate in $\beta OGT^{-/y}$ mice to that of control levels. The mechanism for the beneficial effect of PHZ is unclear, but reduction in ER stress load may contribute to this process. In hyperglycemic states, OGT could also lessen glucose toxicity by increasing O-GlcNAcylation output, thereby reducing the amount of glucosamine and O-GlcNAc in the milieu. OGT can independently modulate proteins (i.e., CamKIV, Pdx-1, and MafA) that regulate β cell failure in glucotoxic conditions (Copeland et al., 2008; Gao et al., 2003; Song et al., 2008b; Sugiyama et al., 2011; Vanderford et al., 2007). Under low glucose levels, MafA and Pdx-1 are localized in the cytoplasm and are transported to the nucleus after high-glucose-induced O-GlcNAcylation. Glucotoxic conditions greatly diminish protein levels of MafA and Pdx-1 (Robertson, 2004). Although the mechanisms are elusive, the process is thought to first involve the exclusion of these proteins from the nucleus and then their disappearance from the cytoplasm. Therefore, it is possible that OGT deficiency in β cells during glucotoxic conditions potentiates the exclusion of these proteins from the nucleus.

Our findings identified a role of O-GlcNAcylation by OGT in ER stress responses in β cell. As mice lacking OGT age, significant β cell mass loss ensues due to ER-stress-induced apoptosis. Although young and normoglycemic mice initially demonstrated normal β cell mass, ER stress markers are increased in islets of βOGT-KO mice. Interestingly, reducing ER stress by deleting one allele of Chop was sufficient to reduce hyperglycemia, improve glucose tolerance, and restore β cell mass in $Chop^{+/-};\beta OGT^{-/y}$ compared to $Chop^{+/+};\beta OGT^{-/y}$ mice. Although insulin level was comparable between $Chop^{+/-};\beta OGT^{-/y}$ mice and controls ($Chop^{+/-};OGT^{x/y}$ or $Chop^{+/+};OGT^{x/y}$), it was not sufficient to normalize random blood glucose level, and it is possible that complete deletion of Chop could result in a complete rescue of β cell function (Song et al., 2008a). It is of high interest in the future to identify which protein(s) involved in the unfolded protein response are regulated by OGT. Remarkably, Jang et al. just reported that eukaryotic translation initiation factor 2α (eIF2α), a major branch of the UPR, is O-GlcNAcylated at Ser219, Thr239, and Thr241, which leads to reduced phosphorylation of eIF2α at Ser51 and ER-stress-induced Chop activation in liver tissue (Jang et al., 2015). Thus, OGT could regulate ER stress by modulating function of genes involved in UPR as shown in Hek293 cells, COS-7, and neonatal rat ventricular myocytes (Ferrer et al., 2014; Jang et al., 2015) as well as ER homeostasis by regulating proteins involved in calcium handling as demonstrated in the heart tissue (Johnsen et al., 2013; Rengifo et al., 2007). Although the exact mechanisms contributing to ER stress in βOGT-KO mice are not fully mapped out, it is possible that loss of OGT in β cells alters ER homeostasis by directly affecting calcium channels or in conjunction with alteration in UPR proteins responses. The prosurvival effects of OGT on ER stress responses observed in βOGT-KO mice support earlier studies by other groups. Using C. *elegans* model, Denzel et al. showed that HBP metabolites enhance protein quality control (Denzel et al., 2014). In the heart and breast cancer cell lines, OGT signaling has been shown to prevent ER stress (Ngoh et al., 2009; Ferrer et al., 2014). Together, the present study supports these findings and extends the setting in vivo and provides a mechanism in glucose-sensitive and ER-stress-prone β cells.

In summary, our data propose OGT as a prosurvival nutrient sensor, modulating proximal insulin signaling and targeting specific downstream pathways to elicit diverse physiological responses and fine-tuning mechanisms governing β cell mass and function. As a highly expressed nutrient sensor in β cells, OGT is poised to regulate cell proliferation, survival, and function. The coordinated actions of OGT, the enzyme that rivals hundreds of kinases involved in phosphorylation protein modification, at multi-levels to regulate intracellular signaling pathways in response to nutrient changes in β cells are just beginning to unravel, and significant advances in our understanding of OGT action underscore the contribution of O-GlcNAcylation in ER stress, Akt signaling, and the etiology of type 2 diabetes.

EXPERIMENTAL PROCEDURES

Generation of O-linked GlcNAc Transferase Knockout Mice

To generate mice with conditional OGT deletion in β cells (herein referred to as male $\beta OGT^{-/y}$ [RIPCre-$OGT^{flox/y}$] or female $\beta OGT^{-/-}$ [RIPCre-$OGT^{flox/flox}$]), we crossed the following breeders: female $OGT^{flox/flox}$ and male mice harboring one allele of Cre-recombinase under the rat insulin 2 promoter (RIPCre) (Figure S1C, RIPCre, provided by Dr. Pedro Herrera, University of Geneva Faculty of Medicine, Geneva, Switzerland) or the inducible mouse insulin promoter (MIPCre-ERTM, Dr. Louis H. Philipson, University of Chicago). Homozygous female $\beta OGT^{-/-}$ was generated by breeding female $OGT^{flox/flox}$ and male RIPCre-$OGT^{flox/y}$. Littermate offspring with or without the RIP or MIPCre transgene were analyzed. Schematic diagram of breeding is shown in Figure S1A. C57BL/6, $OGT^{flox/flox}$, and $Chop^{-/+}$ animals were purchased from Jackson Laboratory. The generation of Akt transgenic mice [caAkt(Tg)] was previously described (Bernal-Mizrachi et al., 2001). All procedures were performed in accordance with the University of Michigan Animal Studies Committee.

Islet Isolation

Primary mouse islets from young and normoglycemic male $\beta OGT^{-/y}$ or female $\beta OGT^{-/-}$ mice were isolated using collagenase (1 mg/ml, Roche Applied Science) and hand picked after filtration with a 70-μm cell strainer (BD Falcon) as described previously (Alejandro et al., 2014).

Western Blotting

Immunoblotting was performed as described previously (Alejandro et al., 2014). Briefly, islet cells were washed after treatments with PBS before adding cell lysis buffer (Cell Signaling Technology) with protease inhibitor cocktail and phoshopStop tablets (Roche Applied Science). Primary antibodies against OGT, O-GlcNAc, and Vinculin were from Abcam. Pdx-1 antibody was purchased from Millipore. Phosphorylated S6 (Ser240), GSK3β (Ser9), and Akt (Ser473, Thr308) and total Akt antibodies were from Cell Signaling. Antibodies against β-actin and tubulin were from Sigma and Chop was from Santa Cruz

Biotechnology. Caspase-12 was from Bioscience. Pro-insulin antibody is from ALPCO. Bip antibody was generated by P.A.'s laboratory.

β Cell Mass, Proliferation, and TUNEL Analysis

Formalin-fixed pancreas tissues were embedded into paraffin. Sections were deparaffinized, rehydrated, and incubated with blocking solution as previously described (Bernal-Mizrachi et al., 2001). Sections were incubated overnight at 4°C with antibodies against insulin (Dako), OGT (Abcam), followed by secondary antibodies conjugated to FITC and Cy3 (Jackson ImmunoResearch). DAPI-containing mounting media (Vector Laboratories) was added to coverslips. β cell mass analysis entails assessing total pancreas and insulin-positive cell areas from five insulin-stained sections (5 μm) separated 200 μm were measured by using Image Pro Software (Media Cybernetics). β cell mass (average β cell fraction multiplied by pancreas weight) assessment was performed using Surveyor Software (Objective Imaging) automated scanning with a Leica fluorescent microscope (Leica Microsystems). Cell proliferation and apoptosis were analyzed using co-staining of Ki67 or TUNEL with insulin on tissue sections of control and knockout mice. At least ~3,000 stained cells were counted from each animal.

Mouse Pancreas Confocal Imaging and Proinsulin Analysis

Pancreata from 4-week-old male $\beta OGT^{-/y}$ and control were prepared as mentioned above but deparaffinized and stained as previously described (Haataja et al., 2013; Wright et al., 2013). For quantification of wild-type proinsulin localization, sections were immunostained with anti-proinsulin (ALPCO), anti-calnexin, and anti-insulin antibodies, and a blinded reader scored the localization of proinsulin in β cells as either a predominant juxtanuclear crescent of increased intensity (Golgi) or mainly diffused and colocalized with calnexin (ER) (Wright et al., 2013). An average of 715 insulin-positive β cells (approximately six islets) was analyzed per genotype.

Glucose and Insulin Tolerance Tests

Fasting glucose levels were measured after an overnight fasting. Glucose tolerance tests were performed by intraperitoneal delivery of 2 g/kg glucose to mice after 12 hr of fasting. Insulin tolerance test was done with 0.75Unit/kg insulin (Humalog, Eli Lilly) to mice after 6-hr fasting. Blood glucose was monitored for 2 hr after glucose or insulin injection. Plasma insulin levels were measured using ultra-sensitive mouse insulin ELISA kit (ALPCO).

Measurement of Intracellular Ca^{2+}

Isolated islets were allowed to recover overnight from islet isolation prior to measurement of intracellular Ca^{2+}. Islets were preloaded with 2.5 μM Fura2-AM (Molecular Probes) in 0.1% DMSO for 30 min at 37°C and washed for 10 min before recording. The recording solution contained, in mM 145 NaCl, 5 KCl, 1.2 $MgCl_2$, 2.6 $CaCl_2$, 10 HEPES, and glucose as indicated (pH 7.4). Ca^{2+} is reported as the ratio of alternative excitation at 340 nm/380 nm, with emission measured at 510 nm (Merrins et al., 2013).

Phloridzin Transplantation Studies

We utilized ALZET Osmotic Pumps (Model 2006, pump flow rate = 0.15ul/hr) to continuously deliver a dose of 0.8 mg/kg/day (~5 μg/μl) of Phloridzin (Sigma) for 25 days in two cohorts of $\beta OGT^{-/-}$ and littermates control: cohort 1 was 70-day-old mice with an average body weight of 25.0 g. Cohort 2 was 50-day-old with an average weight of 20.5 g. Mice were anesthetized with intraperitoneal injection of ketamine (100 mg/kg body weight) and xylazine (10 mg/kg body weight), and ALZET pumps were implanted subcutaneously to all mice. Mice were fully recuperated from the surgery before the in vivo experiments were performed.

Transmission Electron Microscopy

Philips CM-100 electron microscope at 60 kV was utilized to examine ultra-thin sections (70 nm) of isolated islets fixed with 2.5% glutaraldehyde in 0.1 M Sorensen buffer, post-fixed in OsO_4, and stained with 3% uranyl acetate. Megaplus camera system was operated using AMT software (Advanced Microscopy Techniques).

Statistical Analysis

Data are presented as mean ± SEM. Data were analyzed using non-parametric Mann-Whitney tests (U test) using GraphPad Prism v.6 (GraphPad). Results were considered statistically significant when the p value was <0.05.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.11.020.

AUTHOR CONTRIBUTIONS

E.U.A. conceived of, designed and performed experiments, analyzed data, interpreted results, and wrote and edited the manuscript. N.B., S.A., D.K., H.L., L.H., and S.V. performed experiments and analyzed data. L.S.S. and P.A. contributed to discussions. E.B.-M. conceived of the project, interpreted results, and reviewed and edited the manuscript.

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